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Characterization of Insoluble Organophosphate Degrading Bacteria Isolated from the Root of Citrus Plant

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ABSTRACT

Aim: To degrade the water insoluble toxic and non-toxic organophosphate compounds into non-toxic water soluble compounds. Plants consume water soluble components from the soil as nutrients.

Methods: Dilution plate technique with modified Pikovskaya media has been used to isolate the organophosphate degrading bacteria. Three different water insoluble toxic organophosphate insecticides such as chlorpyrifos, methyl-parathion, phorate and two different water insoluble non-toxic organophosphate compounds present in soil such as calcium phytate, lecithin have been used for this study. Organophosphate solubilizing efficiency test, biochemical characterization, antibiotic test, 16S rDNA sequencing, phylogenetic analysis, LC-MS analysis and biofertilization test have been performed.

Results: A potent organophosphate degrading bacteria has been identified as *Enterobacter aerogenes* strain STLR-I on the basis of NCBI database. The accession number provided by GenBank is KX352268. We have named the bacteria as Rz311. R_Z3_{11} can degrade calcium phytate, chlorpyrifos, methyl-parathion and phorate but it cannot degrade soy-lecithin. Biochemical test and antibiotic test have been shown in the table. The LC-MS data shows the biodegradable compounds present in the media are ammonium polyphosphate, ammonium phosphate, P_2O_5 and hypophosphite ion, malic acid, acetic acid, phosphoric acid, gluconic acid etc. The significant vigor index with higher germination percentage of *Cicer arietinum* seeds have been found for all types of treated inoculum.

Discussion: It has been identified as a potent organophosphate biodegrader and bioremidiater with significant biofertilization activity.

Conclusion: R_Z3_{11}, can be used to degrade insoluble toxic and non-toxic organophosphate compounds from environment. The degraded compounds are consumed by the plants for growth.

Key Words: Organophosphate, Biodegradation, Enterobacter, Biofertilizer, LC-MS

INTRODUCTION

Phosphorus is an essential macro-nutrient for the growth of plant. It has important role in various metabolic processes, such as photosynthesis, respiration, energy transfer, signal transduction process, fixation of nitrogen in nodules and others [1-3]. Both inorganic and organic forms of phosphorus are present in the soil. It has been reported that 20-80% of organic phosphorus is water insoluble and plant cannot consume this one[4]. It has also been reported that plant-root can only absorb 0.1% of total phosphorus contained in the soil[5]. Hence, phosphorus deficiency has been supplemented with synthetic chemical fertilizers for green revolution. Along with synthetic phosphate fertilizer, other organophosphates, such as pesticides, insecticides, and acaricides are used for protection of crops. Such organophosphate compounds are toxic in nature. These toxic compounds damage the soil quality, plant growth rate and microbial biodiversity[6-8]. According to the report, crop-yield is reduced by 45% due to the excessive application of pesticides[9].

The isolated bacterium, $R_Z3_{11,}$ has been applied to degrade five different types of organophosphate insecticide compounds. Among the five compounds, three compounds are toxic and two are non-toxic compounds. The soil contains two non-toxic organic phosphate compounds such as calcium phytate and lecithin. Three toxic organo-phosphorus insecticides are generally used in the agricultural field. Thus, in our

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experimental studies, five different water insoluble organic compounds, such as calcium phytate [calcium salt of inositol hexaphosphoric acid], soy-lecithin [2-(nonanoyloxy)-3-(octadeca-9,12-dienoyloxy)propyl 2-(trimethylammonio) ethylphosphonate], methyl-parathion [O, O-dimethyl-O-p-nitrophenylphosphoro-thioate], chlorpyrifos [o,o-diethyl o-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] and phorate [O, O – (diethylthio) methylphosphoradiothioate] are used. Among them chlorpyrifos, methyl-parathion and phorate are applied as pesticides or insecticides. They are neurotoxins and contaminate environment due to the regular fashion of application. Again calcium phytate acts as anti-nutritional factor due to its chelation effects with most of the metals[10]. Global ecology is disturbed with the effect of mineral deficiency and its inhibition of major enzymatic activity [10].

In this investigation, the primary objective is to isolate a potentially environment friendly "microphos" from rhizospheric region of the root of citrus plants. As micro-organisms is one of the cost-effective means for biodegradation of insoluble organophosphate into its soluble form, it is a sustainable approach for plant's phosphorus supply.

Materials and Methods

All the experiments have conducted with sterile double distilled water. All the chemical reagents, which are products of the companies: HIMEDIA/MERCK, India, have been used.

Collection of sample

The sample of citrus plant-root has been collected from Debra, West Midnapore, having latitude of 22.3690° N and longitude of 7.5544° E.

Isolation of organic phosphate solubilizing bacteria

One gram of rhizopheric soil sample of citrus plant has been dissolved in 100 ml of sterilized double distilled water. It is serially diluted from 10^{-1} to 10^{-10} times. 100 µl of such sample from each of one then has been spread on the petri-plate containing modified Pikovskaya media [11-12]. The composition of modified Pikovskaya media is same with the authentic one except calcium tri phosphate. Here, calcium phytate has been used instead of calcium tri-phosphate. Ten different isolates having clear zone surrounding single colonies have been isolated and inoculated in Luria Bertani (LB) broth. Broths have been incubated at 35^0 C for 3 days for bacterial growth. These bacterial cultures have been further tested for degradation of soy-lecithin, chlorpyrifos, methyl-parathion and phorate. Finally the most potent strain has been selected on the basis of five different water insoluble organic phosphate solubilization activity.

Organic phosphate solubilizing efficiency test

The marked bacterial culture has been spot plated on five different petri-plates containing modified Pikovskaya media with five different organic compounds. Here, calcium tri phosphate has been replaced from modified Pikovskaya media by any one of these five organic compounds separately. The five plates have been incubated at 35^0 C for 4 days. The clear zone, from each of the five plates, has been noted for its efficiency test. Insoluble phosphate solubilization efficiency has been calculated using the following formula[13-15]:

Solubilization Efficiency = (solubilization diameter/ growth diameter) X 100

Biochemical characterization

The supernatant or the whole cell bacterial culture has been used for its biochemical characterization according to the Bergey's Manual of Determinative Bacteriology [16] except HCN and siderophore test.

Siderophore and HCN test

The seventy-two hours' bacterial culture has been grown in King's B broth [17] and it has been used for siderophore and HCN test. The protocol of Reeves *et al*. has been followed for siderophore test and dihydroxy benzoic acid has been used for standard curve[18]. HCN test for the isolate has been conducted according to the Reddy et al.[19].

Antibiotic test

100 µl of bacterial culture has been spread on plate containing Nutrient agar media and 20 types of Icosa G-I plus antibiotic disc made by HIMEDIA Pvt. Ltd., Mumbai in sterile condition. All the plates have been incubated at 35^0 C for overnight.

Genomic DNA isolation and 16S rDNA amplification

Bacterial genomic DNA has been isolated according to the Murray *et al.* [20] and it has been dissolved in 50 µl TAE buffer. Here lysozyme is not used as the bacterial culture which is Gram negative in nature. This isolated DNA has been further used for polymerase chain reaction (PCR). The composition of master mix for PCR reaction has been used as 10X 2.5 µl of Taq polymerase buffer, 1 U Taq polymerase enzyme produced by Bangalore Genei, 2 mM of $MgCl_2$, 200 mM of each of deoxynucleoside tri-phosphate, 50 mM of Tris-HCl, 50 ng of genomic DNA, 0.4 µM of each of forward (27F: 5'- AGAGTTTGATCCTGGTCAGAACGCT - 3') and reverse (1492R: 5'- TACGGCTACCTTGTTA CGACTTCACCCC-3') universal 16S rDNA primers. Here Mastercycler personal-22331 of Eppendrof AG, Germany has been used with standard thermal cycling conditions for amplification. The final PCR product has been assayed through 1% agarose gel electrophoresis.

16S rDNA Sequencing and BLAST analysis

Standard Sanger dideoxy method has been followed for 16S rDNA sequencing process. The final sequence has been further used for nucleotide BLAST2.3.1+ program and the top most hits have been selected from the result.

Submission in NCBI Database

The newly identified 16S rDNA sequence has been submitted to GenBank under NCBI database (http://www.ncbi.nlm.nih.gov).

Phylogenetic Tree Analysis

With maximum identity value from different genus have been selected from NBLAST result and has been further used for phylogenetic tree analysis. In this case, MEGA 6.0 program has been used with 26 sequences (collected from BLAST result) having maximum identities with newly identified one. CLUSTALW 1.6 has been used for multiple sequence alignment in MEGA 6.0. Finally best model has been selected for construction of phylogenetic tree.

LC-MS (Liquid Chromatography Mass Spectometry) Analysis

6th day's bacterial culture broth of four different kinds of customized Pikovskaya media (mentioned earlier) containing (1) chlorpyrifos (2) methyl-parathion (3) phorate (4) calcium-phytate as a sole source of phosphate have been used for LC-MS analysis. The centrifuged and purified supernatant has been applied for degradation pattern analysis of the organic compounds. Two different types of the columns have been used for LC-MS analysis of the samples. ODS-3 column has been selected for phorate, chlorpyrifos and calcium-phytate containing samples. Intersil C18 column has been utilized for methyl-parathion containing sample. The other conditions have been applied according to the M. K. Harishankar *et. al.*, Jianhua Hao *et. al.*, N. Bano *et. al.* and Ghorbani-Nasrabadi *et. al.* for chlorpyrifos, methyl parathion, phorate and calcium phytate respectively[21-24].

Bio-fertilization test

Bio-fertilization activity of the strain has been studied from three different types of experimental conditions. In the first set, surface sterilized *Cicer arietinum* seeds have been inoculated in seven days' treated sterilized soil. In the second set, treated seeds have been inoculated in organic phosphate containing treated soil. In the third set, treated seeds have been inoculated into organic phosphate and bacteria containing soil. On the 2nd day, number of seeds germinated has been noted and on the 14th day root-shoot development and other parameters have been noted accordingly.

RESULTS

Each of the experiments has been conducted thrice and their average value is considered for experimental analysis. The name of isolated bacterial strain is R_Z3_{11}.

Organic phosphate (OP) solubilization efficiency test result

Total ten isolates have been identified from Pikovskaya media with calcium phytate containing plate. Out of the ten, only one bacterial strain R_Z3_{11} can solubilize five different insoluble organic phosphate compounds. Organic Phosphate solubilization efficiency has been enlisted in Table 1.

Biochemical characterization

The different biochemical tests of R_Z3_{11} have been performed to study its characteristics. The test results related to gram staining, reaction of citrate, methyl-red, voges-proskaur and oxidase, utilization of catalase, starch hydrolysis, reduction of nitrate and HCN, urease activity, ammonia test and production of indole acetic acid have been shown in Table 2.

Siderophore test result

511.6 ppm of siderophore production with respect to the standard curve has been recorded for this new strain.

Antibiotic test report

The clear zone surrounding each of the 15 antibiotic discs have been shown either the zone of susceptibility or the intermediate on the basis of zone diameter record. On the other hand, 5 (Clindamycin 2, Teicoplanin 10, Vancomycin 30, Linezolid 30 and Erythromycin 15) antibiotic discs have not shown any zone surrounding the antibiotic disc. Clarithromycin 15, Oxacillin 1, Ampicilin 30 and Methicillin 5 are fallen under resistant zone. Azithromycin 15 is fallen under intermediate zone. Gentamicin 10, Ampicillin 10, Amikacin 30, Novobiocin 5, Tetracycline 30, Cephalothin 30, Ofloxacin 5, Co-Trimoxazole 25, Chloramphenicol 30 and Penicillin 10 are under susceptibility zone. Table 3 shows the detailed results.

Nucleotide BLAST results

From the analysis, it has been found that R_Z3_{11} is 99% identical with *Enterobacter aerogenes* strain KCTC with maximum score of 1459. The E-value of it is found to be 0.0.

NCBI database and Phylogenetic tree analysis

GenBank has assigned 16S rDNA partial sequence in its database on 22nd June, 2016. The GenBank has provided accession number of KX352268 for R_Z3_{11}. It has been named as *Enterobacter aerogenes* strain STLR-I. In the model analysis section, it has been found that "JC+I" can be considered as the best model for construction of phylogenetic tree. Figure 1. shows the phylogenetic relationship among different species from different genus.

LC-MS analysis result

Total Ionization Chromatogram (TIC) of LC-MS results of four different samples containing (1) calcium phytate (IP_6) (2) methyl-parathion, (3) chlorpyrifos, (4) phorate have been shown in the Figure 2, Figure 3, Figure 4 and Figure 5 respectively. The different peaks of each of four samples has been mentioned in the Figures 2, 3, 4 and 5.

Biofertilization activity

Figures 6 and 7 show the vigor index and germination index of *Cicer arietinum* seeds' activities in different inoculants. From the in-vitro test of seed germination and their plant-let growth, it has been found that CAP R_Z3_{11}, CH R_Z3_{11}, CAP C, PH R_Z3_{11}, PA R_Z3_{11}, PH C, Control, PA C and CH C are the types of inoculant in treated soil.

DISCUSSION

It is evident from the water insoluble organophosphate solubilization test that the rhizobacterial strain, R_Z3_{11}, is a very potent calcium phytate, chlorpyrifos, methyl-parathion and phorate biodegreder. Again, 585.37% of solubilization index manifest about its higher rate of phytase activity for degradation of calcium phytate. But it has negative lecithinase activity as it is just unable to degrade soy-lecithin.

Biochemical characterization of R_Z3_{11} manifest that it is able to secrete enzymes, such as urease, amylase, oxidase and catalase. Such properties help this specific strain to survive under adverse conditions and also can be used as a potent bioremidieter because it can convert urea into ammonia and CO_2, starch into maltose, may transport electron to cytochrome C. R_Z3_{11} may also have anti-fungal activity due to the presence of positive siderophore reaction. It can form iron-siderophore complex to form a boundary surrounding it and utilizes as per its requirement in iron limiting condition. Again, R_Z3_{11} has plant growth promoting rhizobacterial activity due to its positive result of siderophore.

R_Z3_{11} is highly sensitive to Clarithromycin, Gentamicin, Oxacillin, Ampicillin, Amikacin, Novobiocin, Tetracycline, Cephalothin, Ofloxacin, Co-Trimoxazole, Chloramphenicol, Methicillin, Penicillin, and Azithromycin, that is, R_Z3_{11} is unable to survive in presence of these antibiotics. Hence, R_Z3_{11} is safe to handle.

Phylogenetic analysis has been shown 0.1% divergence among the studied bacterial species. R_Z3_{11} or *Enterobacter aerogenes* strain STLR-I is closely related to *Enterobacter aerogenes* strain NCTC 10006.

The LC-MS analysis shows that R_Z3_{11} is able to degrade completely calcium phytate, chlorpyrifos, methyl-parathion and phorate. Hence the bacterial degraded of four water insoluble organophosphate compounds have been used for LC-MS study. The bio-degraded products contain ammonium phosphate, ammonium polyphosphate, phosphoric acid, P_2O_5 etc. The final bi-products are water soluble, non-toxic compounds that can be easily consumed by the plants and other micro-organisms. R_Z3_{11} strain completely degrades chlorpyrifos into pyruvic acid through TCP (3, 5, 6-trichloro-2-pyrinidol). This TCP degradation and pyruvic acid formation, is the notable work done and it makes this strain different with respect to other reported *Enterobacter sp.* so far.

The seed germination rate of calcium-phytate and phorate is different from methyl-parathion and chlorpyrifos. For calcium-phytate and phorate the seed germination rate is (R_Z3_{11} + OP + seed) > (OP + seed) > seed. Again for methyl-parathion and chlorpyrifos the seed germination rate is (R_Z3_{11} + OP + seed) > control > (OP + seed). The rate of vigor-index is as follows: (calcium-phytate + R_Z3_{11}) > (chlorpyrifos + R_Z3_{11}) > (calcium-phytate without R_Z3_{11}) > (phorate + R_Z3_{11}) > (methyl-parathion + R_Z3_{11}) > (phorate without R_Z3_{11}) > control.

CONCLUSION

The newly isolated bacterial strain R_Z3_{11} or *Enterobacter aerogenes* strain STLR-I, is highly useful for biodegradation of toxic water-insoluble organophosphorus compounds, such as calcium-phytate, chlorpyrifos, methyl-parathion and phorate into non-toxic water soluble compounds. Thus, such types of bio-inoculants can be used to decontaminate the terrestrial as well as aquatic environment from various types of toxic chemicals. The degraded soluble phosphate compounds are consumed by the plants. These soluble phosphates improve soil quality and growth of plants. These soluble phosphates in the soil can reduce the consumption of synthetic fertilizers. Field application is our future objective to extend this work.

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Conflict of interest

All authors have no conflict of interest in conducting and publishing the research paper.

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Table 1: Organic Phosphate solubilization efficiency on sixth day

No. of Observation	Treatment	Time of Inoculation	Solubilization Efficiency (%) of $R_{23_{11}}$
1	PVK(CAP)	4 days	585.37
2	PVK(L)	4 days	0
3	PVK(PA)	4 days	184.29
4	PVK(CH)	4 days	240
5	PVK(PH)	4 days	186.67

In Table 1. PVK(CAP), PVK(L), PVK(PA), PVK(CH) and PVK(PH) implies modified Pikovskaya media containing calcium phytate instead of calcium tri phosphate, modified Pikovskaya media containing lecithin instead of calcium tri phosphate, modified Pikovskaya media containing methyl-parathion instead of calcium tri phosphate, modified Pikovskaya media containing methyl-parathion instead of calcium tri phosphate, modified Pikovskaya media containing chlorpyrifos instead of calcium tri phosphate and modified Pikovskaya media containing phorate instead of calcium tri phosphate respectively.

Table 2: Biochemical characteristics of R_Z3_{11}

Characteristics	R_Z3_{11}
Gram Staining Reaction	-
Cell Shape	Rod
Citrate Reaction	+++
Methyl-Red Reaction	-
Voges-Proskaur	+++
Oxidase	+/-
Utilization of Catalase	++++
Starch Hydrolysis	+
Nitrate Reduction	-
Urease Activity	+++
Ammonia Test	++++
Indole Acetic Acid Production	++
HCN Reduction	-

In Table 2. the meaning of the symbols are: "-" means negative result, "+/-" means very weakly positive, "+" means weakly positive, "++" means moderately positive, "+++" means strongly positive, "++++" means very strongly positive.

Table 3: Antibiotic disk diffusion test report

Name of antibiotics used (power is in mcg)	Zone of inhibition (mm)
Clarithromycin 15	12
Gentamicin 10	27
Oxacillin 1	10
Ampicillin 10	18
Amikacin 30	27
Novobiocin 5	22
Clindamycin 2	-
Tetracycline 30	27
Teicoplanin 10	-
Vancomycin 30	-
Cephalothin 30	22
Linezolid 30	-
Ofloxacin 5	33
Ampicilin 30	10
Co-Trimoxazole 25	28
Erythromycin 15	-
Chloramphenicol 30	31
Methicillin 5	10
Penicillin 10	21
Azithromycin 15	16

In Table 3. the "-" implies negative result.

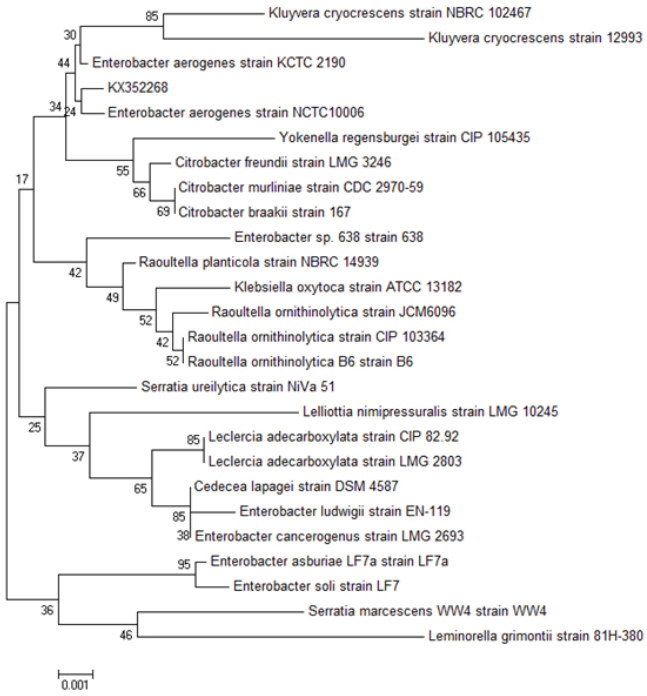

Figure 1: Phylogenetic relationship among different genus having maximum identity score through maximum likelihood analysis. Here KX352268 is the isolated bacterial strain from rhizospheric region of root of citrus plant.

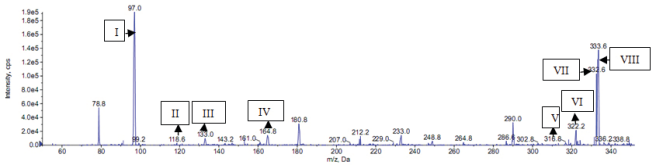

Figure 2: LC-MS spectrum of culture broth containing calcium phytate as a sole source of phosphate. The TIC contains peaks of (I) and (II) Ammonium polyphosphate (M) and (M+Na-2H$^+$), (III) Malic acid (M-H$^+$), (IV) Ammonium phosphate (M+NH_3-H$^+$), (V), (VII) and (VIII) IP_2 (M-H$^+$), (M+NH_3-3H$^+$) and (M+NH_3-2H$^+$) (VI) Phosphorus penta-oxide (M+K-H$^+$).

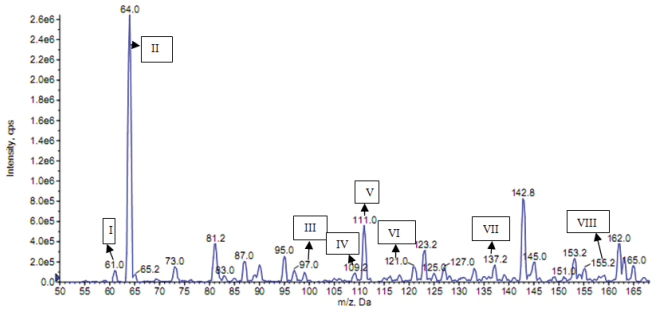

Figure 3: LC-MS spectrum of culture broth containing methyl-parathion as a sole source of phosphate. The TIC contains peaks of (I) Acetic acid (M+H$^+$), (II) Hypophosphite ion (M+H$^+$), (III) Ammonium polyphosphate (M), (IV) & (V) p-Aminophenol (M) & (M+2H$^+$), (VI) & (VII) Phosphoric acid (M+Na) & (M+K), (VIII) Diethylphosphoric acid (M+H$^+$).

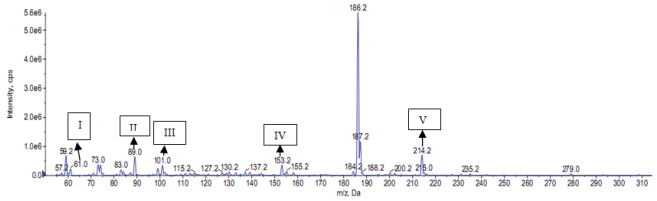

Figure 4: LC-MS spectrum of culture broth containing chlorpyrifos as a sole source of phosphate. The TIC contains peaks of (I) Acetic acid ($M+H^+$), (II) Pyruvic acid ($M+H^+$), (III) Maleamide semialdehyde ($M+2H^+$), (IV) phosphorothioic acid (M+K), (VI) TMP ($M+2H^+$).

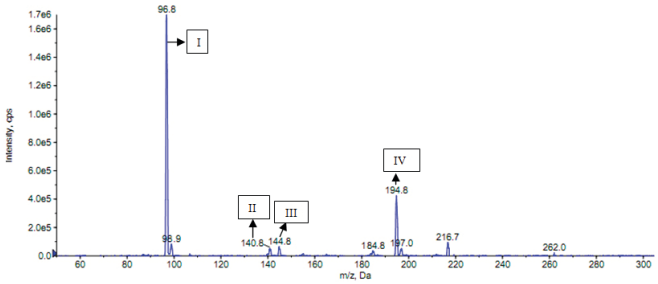

Figure 5: LC-MS spectrum of culture broth containing phorate as a sole source of phosphate. The TIC contains peaks of (I) Ammonium polyphosphate (M), (II) Ethylthiophosphate ($M-H^+$), (III) Ammonium phosphate ($M-4H^+$), (IV) Gluconic acid ($M-H^+$).

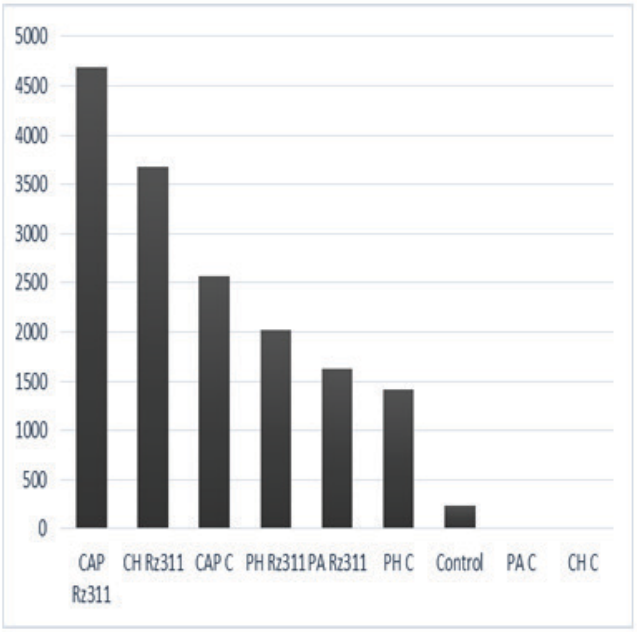

Figure 6: Bar chart representing the vigor index of *Cicer arietinum* plant-lets in different inoculants.

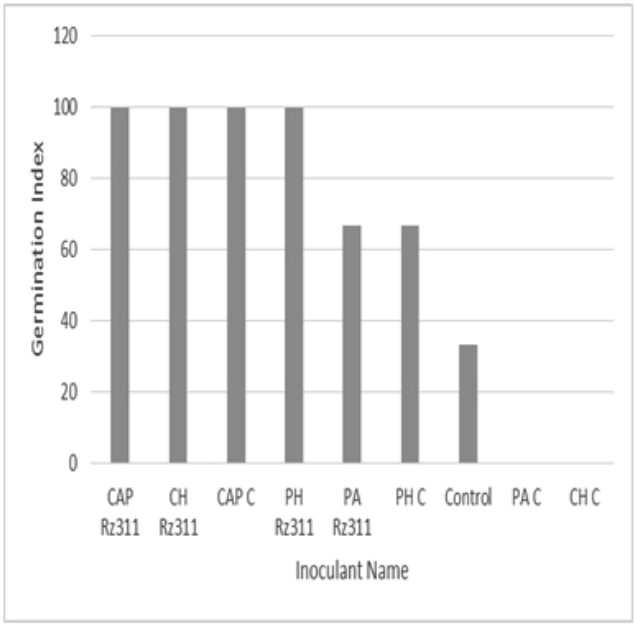

Figure 7: Bar chart representing the germination index of *Cicer arietinum* seeds on 2nd Day in different inoculants.